FEBS Letters 580 (2006) 2465–2470

Exposure to modeled microgravity induces metabolic idleness in malignant human MCF-7 and normal murine VSMC cells

Rita Coinu[b,1], Angela Chiaviello[a,1], Grazia Galleri[b], Flavia Franconi[c], Elvira Crescenzi[a], Giuseppe Palumbo[a,*]

[a] *Dipartimento di Patologia e Biologia Cellulare e Molecolare/IEOS CNR, Università di Napoli Federico II, Via S. Pansini, 5, I-80131 Naples, Italy*
[b] *Dipartimento di Scienze Fisiologiche Biochimiche e Cellulari, Università di Sassari, Sassari, Italy*
[c] *Dipartimento di Scienze del Farmaco, Università di Sassari, Sassari, Italy*

Received 24 February 2006; accepted 27 March 2006

Available online 7 April 2006

Edited by Veli-Pekka Lehto

Abstract **We investigated the effect of modeled microgravity (MMG) on normal vascular smooth muscle cells (VSMC) and neoplastic human breast cancer cells (MCF-7). In both cell types, MMG induced partial arrest in G_2M and increased p14-3-3, HSP70, HSP60 and p21 expression. Cells synchronized by 24 h starvation reentered the normal cycle within 24 h if released in complete medium and exposed to normal gravity, but not if exposed to MMG. Similarly, MMG prevented VSMC and MCF-7 cells from overcoming growth arrest and re-synthesizing DNA. This study shows that cells adjust their metabolic rate in response to MMG.**

Keywords: Modeled microgravity; Cell cycle; Cell metabolism

1. Introduction

Microgravity induces adverse and metabolic effects in animals, humans and cellular systems [1–4]. We have evaluated the effects of microgravity on cell proliferation, glucose uptake, amino acid uptake and incorporation into proteins in neoplastic human breast cancer cells (MCF-7) and normal murine vascular smooth muscle cells (VSMC). Although these cells originate from different tissues and different species and have a distinct differentiation status, both are p53-positive and require similar culture conditions. In addition, both cell types have insulin receptors, which suggest that their metabolic activities depend on culture nutrients, including the insulin normally present in fetal calf serum (FCS). As transformed cells, MCF-7 require constant refurbishment to meet their energy requirements and are hence highly dependent on glucose metabolism for growth, whereas muscle cells depend on amino-acid metabolism (protein synthesis and breakdown). Therefore, glucose uptake and amino acid uptake and utilization studies conducted in these cells under conditions of microgravity may reveal derangements of metabolism induced by space-related stress.

2. Materials and methods

2.1. Cell cultures

Human breast cancer MCF-7 cells were grown in DMEM-5% FCS^+ at 37 °C in a humidified atmosphere as previously reported [5]. VSMC were harvested from aortas of young male rats (Sprague Dawley) by the media explant technique and cultured in DMEM-10% FCS^+ as described elsewhere [6]. Serum starvation (in modified Krebs Ringer phosphate solution in the presence of 0.025% albumin) is designated "FCS^-". [^{3}H]-thymidine, 2-deoxy-D-[^{14}C]-glucose and [^{35}S]-methionine were purchased from Amersham (Milan, Italy). Concentration of proteins extracted from cells was measured as according to Bradford [7].

2.2. Modeled microgravity

Six flasks containing 1.5×10^5 MCF-7 cells or VSMC, at time 0 were filled with FCS^+ media and capped. Three (MCF-7 or VSMC) flasks were placed in a temperature-controlled room (37 °C) on a running random positioning machine (RPM; Dutch Space, The Hague, NL); the remaining three flasks were fasten to the edge of the RPM and served as controls (see below). Samples and controls were left to grow for 48 h in modeled microgravity (MMG).

2.3. Normal gravity

Cells grown on the RPM are subjected to changes in gravity and mechanical stress due to the vibration of the machine. Therefore, we fasten the control flasks to the edges of the RPM. All control cells considered hereafter are those subjected to mechanical stress only.

2.4. DNA synthesis, glucose uptake, methionine uptake and incorporation into neo-synthesized proteins

2.4.1. DNA synthesis. These assays were carried as described previously [5]. MCF-7 or VSMC (normally 1.5×10^5 cells) were incubated with ~1 μCi/ml of [^{3}H]-thymidine as detailed in the legends to Figs. 1B and 4, respectively, then rinsed three times with cold PBS, and fixed in cold 10% trichloroacetic (TCA) acid. The precipitates were washed with ethanol and dried by evaporation. The precipitates were washed and solubilized in NaOH: a portion was used to determine protein concentration [7]; the remaining part was neutralized with HCl and counted by liquid scintillation. Incorporation was calculated as counts per minute (cpm) per mg of protein and expressed as percentage of control. Three separate experiments have performed. Each experiment was done using triplicate samples.

2.4.2. Glucose uptake. VSMC and MCF-7 cells were plated at a density of 1.5×10^5 cells and grown in FCS^+ medium. Cells were cultured for about 24 h, transferred to a temperature-controlled room (37 °C) and exposed to MMG in a RPM for 48 h. They were then washed and starved for 5 h in modified Krebs Ringer phosphate solution. Incubation (37 °C)

*Corresponding author.
E-mail address: palumbo@unina.it (G. Palumbo).

[1] R.C. and A.C. contributed equally to this work.

Abbreviations: MMG, modeled microgravity; VSMC, vascular smooth muscle cells; FCS, fetal calf serum; RPM, random positioning machine; MCF-7, neoplastic human breast cancer cells; NG, normal gravity

0014-5793/$32.00
doi:10.1016/j.febslet.2006.03.078

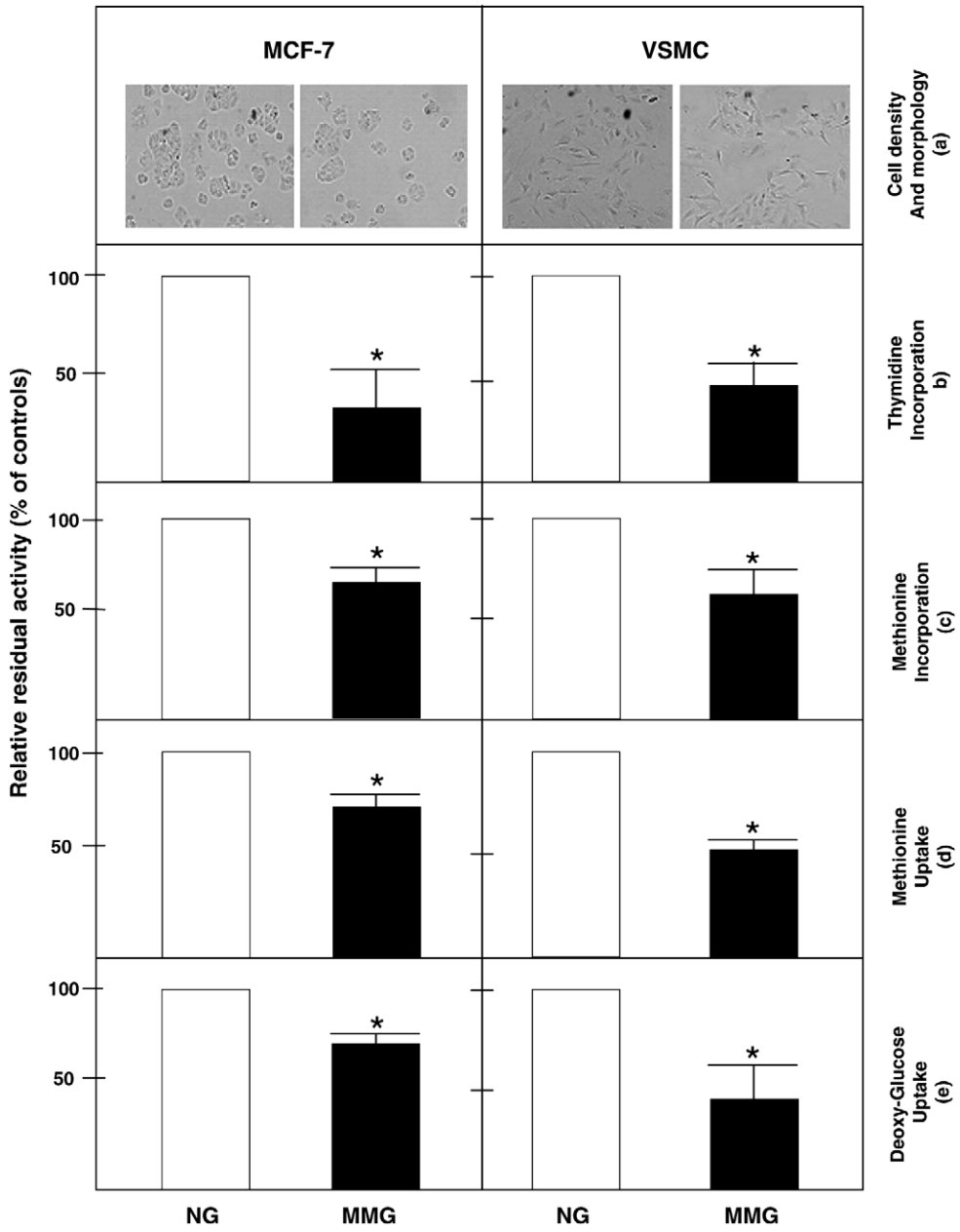

Fig. 1. Panel a: MCF-7 cells (left) and VSMC (right) after 48 h of growth in NG and MMG. Panel b: Incorporation of [^{3}H]-thymidine into DNA in MCF-7 (left) and VSMC cells (right) exposed to MMG (close bars) and NG (open bars). Panels c and d: [^{35}S]-methionine uptake and TCA-precipitable material in MCF-7 (left) and VSMC cells (right) exposed to NG and MMG. Panel e: Uptake of 2-deoxy-[^{14}C]-glucose in MCF-7 (left) and VSMC (right) exposed to NG and MMG. Data are calculated by dividing the measured cpm per mg of protein and were expressed as % of respective controls to allow the results of three experiments to be pooled and analyzed collectively. Statistical significance in each experiment was estimated with the *t* test, where $P < 0.05$ was considered statistically significant.

was continued for 30 min after the addition of 2-deoxy-D-[^{14}C]-glucose and 0.17 mM glucose as carrier [5]. Next, cells were washed and solubilized in NaOH and the protein concentration determined [7]. Finally [^{14}C]-deoxy-glucose radioactivity was counted by liquid scintillation. Uptake was calculated as counts per minute (cpm) per mg of protein and expressed as percentage of control. Three separate experiments have performed. Each experiment was done using triplicate samples.

2.4.3. Methionine uptake and incorporation. Amino acid uptake was determined in triplicate by measuring the uptake of [^{35}S]-methionine under normal gravity (NG) or MMG. After 36 h of MMG, cells were removed from the RPM, washed, repositioned on the RPM, and starved for 12 h with modified Krebs Ringer phosphate solution in the presence of 0.05% albumin. Incubation (37 °C) was continued for 30 min after the addition of [^{35}S]-methionine (12.5 μCi/flask). Next, cells were thoroughly washed and solubilized in NaOH. The solution was divided in three parts: a portion was used to determine protein concentration [7], a portion was used to count total radioactivity (free plus incorporated into proteins), a portion was used to measure protein-associated radioactivity after precipitation with TCA as described elsewhere [8]. Results (counts per minute per mg of total protein) are expressed as percentage of controls. Three similar experiments have performed. Each experiment was done using triplicate samples.

2.5. Statistical analysis and presentation of data

Thymidine incorporation, methionine incorporation/uptake and glucose uptake were expressed as % of respective controls to allow the results of multiple experiments to be pooled and analyzed collectively. Statistical significance was estimated with the T-test. A *P* value of less than 0.05 was considered statistically significant.

2.6. Flow cytometry – polyacrylamide gel electrophoresis and Western blotting

At least 20 000 events were collected for each sample. Samples prepared as described by Crescenzi et al. [5] were routinely run in quadruplicate in an Excalibur Cytofluorimeter (Becton Dickinson, Mountain View, CA).

Total cell protein preparations, the materials and the detailed procedure used for electrophoresis and Western blotting (WB) on nitrocellulose filters, including the antibodies (primary and secondary) are as previously reported [9]. In brief, after staining with Red Ponceau, suitable filters (i.e., those displaying a uniform level of staining in all lines) were developed using an electro chemiluminescent WB detection reagent (Amersham) and quantified by scanning with a Discover Pharmacia scanner equipped with a Sun Spark Classic Workstation. The protein extracts, obtained by three flasks were individually analyzed by WB. Results are representative of three experiments.

2.7. Cell starvation, cycle arrest and recovery

Dishes containing $\sim 4 \times 10^4$ MCF-7 or VSMC cells were incubated for 24 h at 37 °C in 7 ml of complete medium. 24 h after attachment, cells were washed and starved with modified Krebs Ringer phosphate solution in the presence of 0.05% albumin for 24 h, at which time the cell cycle was arrested. To determine the time required for cells to reenter the cycle, cell cycle arrested cells were released in complete medium and analyzed by cytofluorimetry at 4, 8, 12 and 24 h. The effect of MMG on MCF-7 and VSMCs capacity to reenter the cycle, was estimated by cytofluorimetry, whereas the recovery of original proliferation rate, was evaluated by [^{3}H]-thymidine incorporation experiments.

Cell cycle. Recovery after cell synchronization. Synchronized MCF-7 cells (seeded at a density of $\sim 1.5 \times 10^5$ cells/flask) were obtained by starvation in FCS$^-$ medium. Thirty-six hours later, flasks were drained, refilled with FCS$^+$ medium, divided into two groups of three flasks each and exposed, respectively, to MMG or NG (controls) for 24 h. Cells were then fixed and analyzed by cytofluorimetry.

[^{3}H]-thymidine incorporation. Recovery from cell synchronization. To evaluate recovery from cell synchronization, six flasks containing $\sim 2.0 \times 10^5$ attached MCF-7 cells (time 0) were starved for 30 h by exposure to FCS$^-$ for 24 h. Once cells were synchronized, FCS$^-$ medium was replaced with FCS$^+$ medium containing 1 μCi/ml [^{3}H]-thymidine. Three flasks were kept under NG, and three were put on the RPM, and incorporation was measured 15 h later. In a different experiment, six flasks containing $\sim 2.0 \times 10^5$ VSMC cells were released in FCS$^-$ and exposed to MMG for 24 h. Cells were then released in FCS$^+$ medium containing 1 μCi/ml [^{3}H]-thymidine, divided in two groups (three in NG and three in MMG) and incubated for an additional 10 h before final assessment of thymidine incorporation. Each experiment, with MCF-7 and VSMC was performed twice, in identical conditions as above.

Table 1
Cell cycle of MCF-7 cells and VSMC exposed to NG and to MMG for 24 h

	MCF-7		VSMC	
	NG	MMG	NG	MMG
$G_{0/1}$	63.4 ± 0.14	64.4 ± 4.0	70.4 ± 1.5	67.5 ± 1.5
S	27.7 ± 1.4	18.4 ± 2.5	23.7 ± 1.34	17.7 ± 2.2
G_2/M	8.9 ± 1.5	17.2 ± 2.8	5.9 ± 0.14	14.8 ± 0.7

Average of three measurements.

3. Results and discussion

We have comparatively analyzed two cell lines, namely VSMC (normal) and MCF-7 (transformed) cells to understand how and to what extent weightlessness alters cellular properties

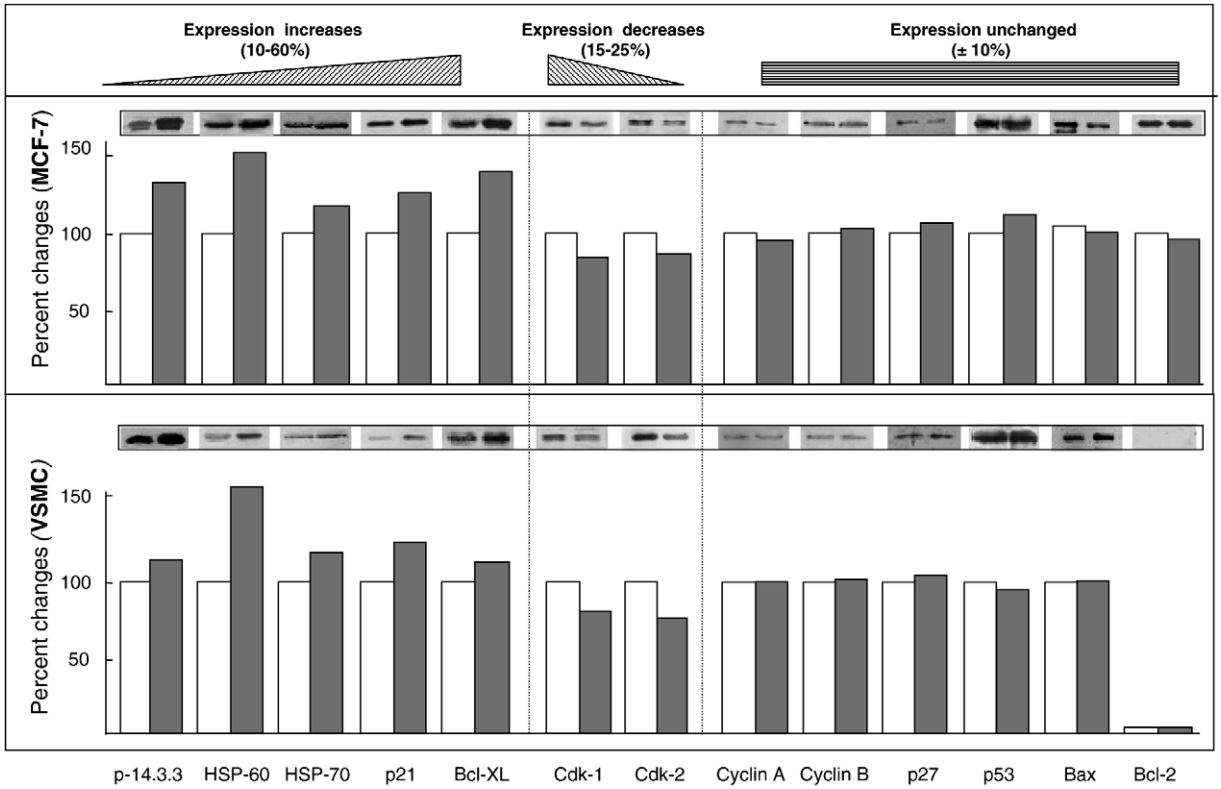

Fig. 2. Each histogram, obtained by digital densitometry, compares the expression of the marked protein in MMG (closed bars) versus the NG conditions (controls, open bars). Density of controls was arbitrarily set at 100. Upper panel refers to MCF-7 cells; lower panel to VSMC cells. Filter staining with Red Ponceau has been used as indicator of uniform protein transfer, while actin (not shown), has been used as loading control. The representative WB inserts show the profile of the indicated proteins in MMG and NG conditions. Although multiple experiments and scans have been performed, all data may be considered only denotative of an apparent increasing (left), decreasing (center) or unchanging (right) trend in the particular protein expression.

including basal proliferation rate (thymidine incorporation), glucose uptake and methionine uptake/incorporation, cell cycle profiles and the expression of several proteins.

3.1. Cell growth and proliferation

Proliferation was much lower in MCF-7 cells and in VSMC exposed to MMG versus NG (Fig. 1, panel a). Moreover, [^{3}H]-thymidine incorporation was significantly ($P < 0.05$) lower in MCF-7 cells (~70%) and in VSMC (~50%) exposed to MMG (Fig. 1, panel b). As found in free-floating Jurkat cells [10], MMG caused both cell types to slightly accumulate in G_2/M (Table 1). This partial remodeling of the cell cycle was associated with reduced cell proliferation. Prolonged MCF-7 mitosis during MMG is not a new finding and has been attributed to weightlessness-induced alteration of microtubules [4]. Also Meyers et al. [11] found that MMG alters proliferation rate, and reported that MMG, by decreasing integrin/MAPK signaling, contributes to reduced osteoblastogenesis during differentiation of human mesenchymal stem cells. Similarly, Plett et al. [12] demonstrated that MMG causes alterations of human hematopoietic progenitor cells.

3.2. Cellular metabolic activity

Fig. 1 shows also the effects in both MCF-7 and VSMC cells of MMG as compared to NG on some metabolic cellular activities. It appears that the incorporation of methionine into TCA-precipitable materials (panels c), its cellular uptake (panels d) and the deoxy-glucose uptake (panels e), are neatly reduced. All the differences noted were statistically significant ($P < 0.05$).

3.3. Protein expression

Protein expression has been quantitated by digital densitometry of three independent experiments. As may be noticed by the bars in Fig. 2 (see additional details in the legend), MMG-exposed MCF-7 cells (upper panel) express increased levels of HSP60 (~50%), Bcl-XL (~35%) and p14-3-3 protein (~30%); even p21 and HSP70 appear to be elevated, although to a lesser extent. Densitometry indicated possibly lower levels of both Cdk-1 and -2 (~15%). Similarly MMG did not (or very imperceptibly) affect the expression of p53, Bax, Bcl-2, p27 and Cyclins or PARP (not shown). As shown in lower panel of the same figure, MMG-exposed VSMC cells appear to express increased levels of HSP60 (>50%) and, to a lesser extent, of p21 (~25%), HSP70 (~20%), p14-3-3 (~20%) and Bcl-XL (~15%). Cyclin kinases appear to decrease of ~20%, while cyclins, p53, p27, Bax and also Bcl-2 (which indeed is already undetectable in control VSMC cells) appear to remain unchanged. Even in this case, PARP was not affected (not shown).

The increased expression of the constitutive proteins HSP-60, in particular, and, to a lesser extent, HSP-70 is obscure,

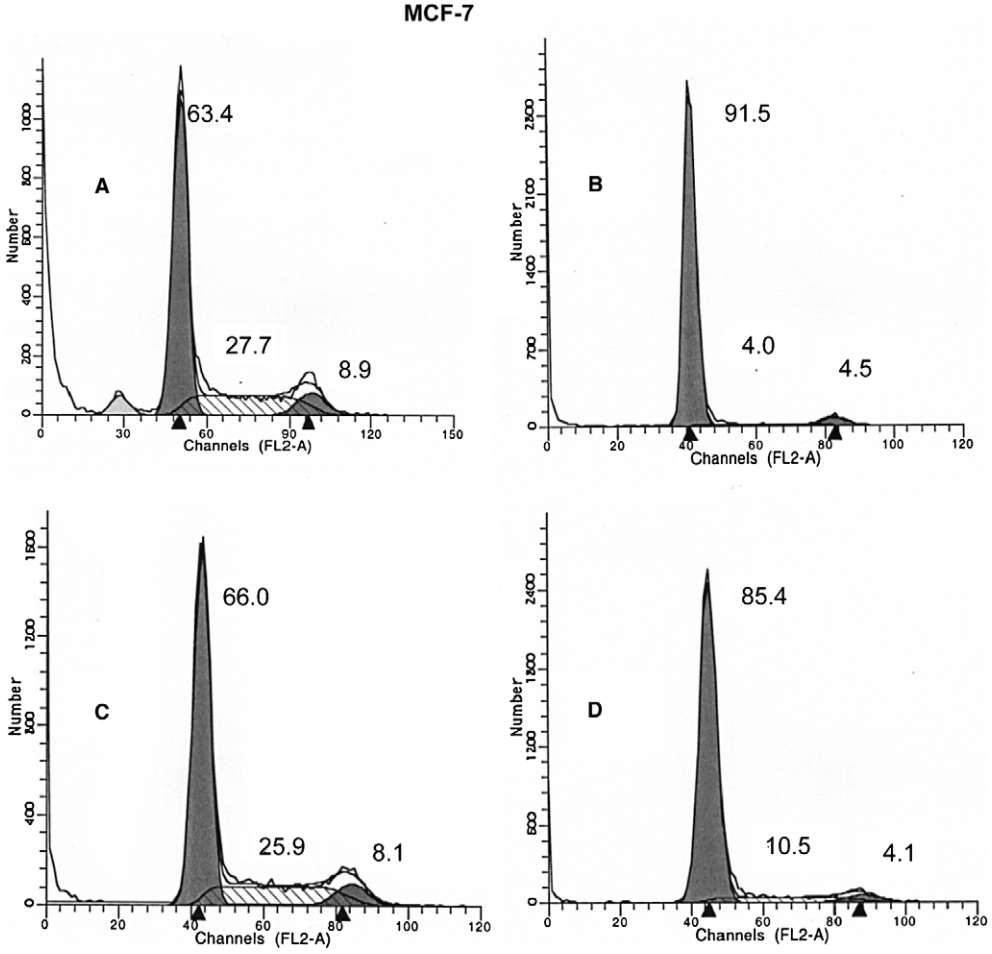

Fig. 3. MCF-7 growing in FCS$^+$ in NG (a); cells starved in FCS$^-$ for 24 h under NG (b); cells starved for 24 h (as before) and released in FCS$^+$ for a further 24 h under NG (c); cells starved for 24 h and released in FCS$^+$ for a further 24 h under MMG (d). Data shown are representative of triplicate samples.

although the two proteins may work in tandem to facilitate the refolding process after damage [13]. It is possible that MMG drives cells beyond their normal activity. This metabolic disturbance, accompanied by a modest, albeit reproducible increase in p21 and Bcl-XL expressions, negatively influences cell proliferation. Similarly, MMG affected appreciably the expression of p14-3-3 (especially in MCF-7). This protein is important for such vital regulatory processes as mitogenic signal transduction and cell cycle control [14]. In agreement with the cell cycle profiles, which lacked signs of apoptosis, the profiles of the pro-apoptotic proteins BAX and PARP (not shown) are unchanged as compared to controls.

Since digital densitometry integrates bands produced on films by chemiluminescent signals, which are intrinsically semi-quantitative, the data should be regarded with some caution: nonetheless, they are not in contrast with our metabolic and cytofluorimetric findings (see below) and previous observations made by others [10,11].

3.4. Effect of MMG on synchronized cells

We used two strategies, both based on a "stop and go" approach, to determine whether MMG affects proliferation rate. This requires preliminary synchronization of cells by serum starvation (*stop*) followed by re-nourishing of cells in FCS-containing medium (*go*) in MMG or NG (Fig. 3, panel a, controls). In one strategy we examined the original cell cycle profile, and in the other we evaluated restoration of the propensity to synthesize DNA by examining [^{3}H]-thymidine incorporation.

Cell cycle. Exposure of MCF-7 cells for 24 h to a FCS$^-$ solution caused cell cycle arrest and complete (~92%) synchronization in G_1 (Fig. 3, panel b). Upon release in FCS$^+$ medium, these cells resumed normal cycling activity, and the original profiles were restored after exposure to NG for 24 h (Fig. 3, panel c). This was not the case of cells exposed to MMG for 24 h. In this case >90% of cells arrested by starvation in G_1 did not progress (Fig. 3, panel d). This result indicates that MMG can hamper the normal cycling of the malignant fast-proliferating MCF-7.

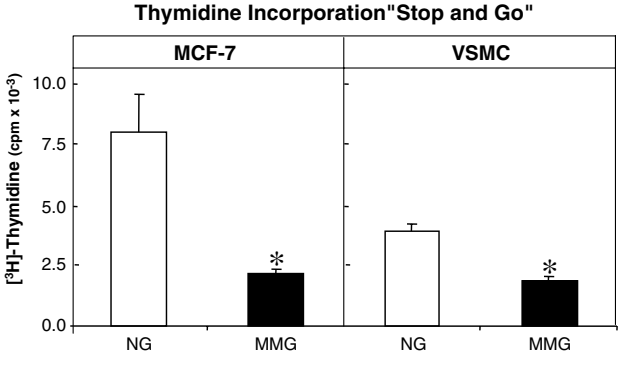

Fig. 4. Stop and go experiments. Two separate experiments have been performed on both cell type (MCF-7, left; VSMC, right). Each individual experiment was performed using three samples and three controls. The data reported in the chart depict the best estimate (weighted average) of each pair of experiments. Initial cell synchronization was obtained by serum deprivation (stop). [^{3}H]-thymidine incorporation was measured following release of cells in FSC$^+$ medium (go). Open bars refer to NG and close bars to MMG. The observed differences were statistically significant ($P < 0.05$). See Section 2 for further details.

[^{3}H]-thymidine incorporation. To determine whether MMG affects DNA synthesis, we examined fast growing MCF-7 cells and the normal slow proliferating VSMC. MCF-7 cells were synchronized by simple starvation for 24 in NG, whereas the slow proliferating VSMC cells were synchronized by starving cells exposed to MMG for 24 h.

The best estimates of two experiments, based on the weighted average of the determinations are reported in Fig. 4, indicated that DNA synthesis was reduced by ~4-fold (MCF-7, left panel) and ~2-fold (VSMC, right panel) in cells exposed to MMG. In both cases, the differences of the means are statistically significant ($P < 0.05$).

In conclusion, our results show that MMG alters the cytofluorimetric profile and slows down fundamental metabolic activities (glucose uptake, methionine uptake/incorporation and thymidine incorporation) in normal and transformed cells. MMG did not affect significantly the expression of most proteins that are related to the cell cycle and apoptosis, whereas it altered the expression of stress proteins. The large increase in the expression of HSP-60 and a minor increase in HSP-70 and 14-3-3 protein, suggest that transformed and normal cell lines are able to "sense" the altered conditions of gravity. We hypothesize that cells respond to stress by putting the proliferative and metabolic machinery on "stand by". MMG-promoted quiescence is demonstrated in both cell lines by the cell cycle profiling data and thymidine incorporation after synchronization. MMG alters the hydrodynamics of nutrient fluids (buoyancy and convection), that, in turn, affects the cell environment and cell–cell interactions. These changes, responsible for previously reported alterations in cell morphology [4,10] and in metabolic activity in animals [1] may explain also our results. It remains to be established whether or not these effects are fully or partially reversible, if are transient and if they may represent potential danger to human health in space, particularly in the presence of other hazards such as cosmic radiation.

Acknowledgements: G.P. is personally indebted to the Responsible of the RPM facility in Sassari for his generosity. All Authors thank Gavino Campus for the skilled technical support, Jean A. Gilder for text editing and the MoMa project (ASI, Rome, Italy) for funding.

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